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The NS5A-binding heat shock proteins HSC70 and HSP70 play distinct roles in the hepatitis C viral life cycle ☆

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ABSTRACT

We previously identified HSP70 and HSC70 in complex with NS5A in a proteomic screen. Here, coimmunoprecipitation studies confirmed NS5A/HSC70 complex formation during infection, and immunofluorescence studies showed NS5A and HSC70 to colocalize. Unlike HSP70, HSC70 knockdown did not decrease viral protein levels. Rather, intracellular infectious virion assembly was significantly impaired by HSC70 knockdown. We also discovered that both HSC70 nucleotide binding and substrate binding domains directly bind NS5A whereas only the HSP70 nucleotide binding domain does. Knockdown of both HSC70 and HSP70 demonstrated an additive reduction in virus production. This data suggests that HSC70 and HSP70 play discrete roles in the viral life cycle. Investigation of these different functions may facilitate developing of novel strategies that target host proteins to treat HCV infection.

Introduction

The hepatitis C virus (HCV) infects 3% of the world population and is mainly responsible for liver transplantation in patients with cirrhosis in developed countries (Shepard et al., 2005). Furthermore, HCV is the most common chronic blood borne pathogen in the United States (US) affecting 1.8% of the population and is the major etiologic factor responsible for the recent doubling of hepatocellular carcinoma (HCC) (El-Serag, 2002).

HCV infection is currently treated by the combination of pegylated interferon-α (PEG-IFN), ribavirin, and the recently approved NS3/4A protease inhibitors which have led to an increased sustained

Abbreviations: FLuc, *Firefly* luciferase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HCVcc, HCV cell culture; HSC70, heat shock cognate protein 70; HSP, heat shock protein; HSP70, heat shock protein 70; IRES, internal ribosomal entry site; MBP, maltose binding protein; NBD, nucleotide binding domain; NCR, non-coding region; NS, non-structural; NS5A, non-structural protein 5A; ORF, open reading frame; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PEG-IFN, pegylated interferon-α; RLuc, *Renilla* luciferase; SBD, substrate binding domain; SPR, surface plasmon resonance; SVR, sustained virological response

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virological response (SVR) compared with PEG-IFN and ribavirin alone, but also increased adverse events including anemia and gastrointestinal symptoms (Ciesek and Manns, 2011). Nevertheless, a significant number of patients cannot receive these treatments as they require PEG-IFN. For these reasons, there is the need to develop adjunct or replacement therapies that are less toxic and more efficacious in terms of higher SVR rates and HCC prevention.

HCV is an RNA virus classified in the genus *Hepacivirus* in the *Flavivirus* family and possesses an approximately 10 kb positive sense RNA genome. The 5' non-coding region (NCR) of the viral genome possesses an internal ribosomal entry site (IRES) (Wang et al., 1993), a *cis*-acting element found in some host RNA transcripts as well as in viruses that allow ribosomal translation initiation to occur internally within a transcript *in lieu* of 5' cap dependent translation (Pacheco and Martinez-Salas, 2010). The HCV viral life cycle in a cell can be divided into six phases: (1) binding and internalization, (2) cytoplasmic release and uncoating, (3) viral polyprotein translation and processing, (4) RNA genome replication, (5) packaging and assembly, and (6) virus morphogenesis and secretion (Moradpour et al., 2007).

The viral non-structural protein 5A (NS5A), a 56–59 kDa phosphoprotein, is a multi-functional protein and a component of the viral replicase complex. It has been implicated in regulation of HCV genome replication, IRES-mediated viral protein translation, virion assembly, and infectious virion secretion (He et al., 2003; Hughes et al., 2009; Khachatoorian et al., 2012a, 2012b; Tellinghuisen et al., 2008).

Interestingly, cellular heat-shock proteins (HSPs) play an important role in the replication of RNA viruses (Vasconcelos et al., 1998; Weeks and Miller, 2008; Zheng et al., 2010). HSPs, which normally assist unfolded or mis-folded polypeptide chains to (re)fold into functional proteins, are crucial for cell survival during stressful conditions (Mayer and Bukau, 2005). We have previously identified, through coimmunoprecipitation and subsequent mass spectrometric analyses, an NS5A/HSP complex composed of NS5A, HSP70, and HSP40 (cofactor of HSP70) and demonstrated their colocalization in huh-7 cells (Gonzalez et al., 2009). We further showed that both NS5A-augmented IRES-mediated translation and virus production are blocked by (1) HSP70 knockdown, (2) the HSP synthesis inhibitor quercetin, a bioflavonoid, and (3) a small peptide from NS5A domain I that is capable of blocking NS5A/HSP70 interaction, with no associated cytotoxicity (Gonzalez et al., 2009; Khachatoorian et al., 2012a, 2012b). These findings support our hypothesis that HCV utilizes an NS5A/HSP complex to facilitate IRES-mediated translation of its genome.

Our mass spectrometric analyses also identified heat shock cognate protein 70 (HSC70) in complex with NS5A (Gonzalez et al., 2009). HSC70, a crucial housekeeping gene, is the constitutively expressed homolog of HSP70 that bears significant (86%) homology to HSP70 (Daugaard et al., 2007). It has been reported that HSC70 is a component of the viral particle and its downregulation significantly reduces virus production (Parent et al., 2009). Either modulation of assembly or viral secretion was implicated in this report.

In this study, we sought to further analyze the binding of HSC70 and NS5A biochemically and to examine various phases of the viral life cycle to identify the specific step(s) that requires HSC70 during viral proliferation.

Results

HSC70 directly binds NS5A in vitro

In our previous report, we described the identification, through mass spectrometric analyses, of a number of host HSPs, including HSP70 (specifically the HSPA1A gene) and HSC70 (HSPA8 gene), in complex with the viral NS5A protein (Gonzalez et al., 2009). We have also reported that HSP70 directly binds NS5A through a hairpin peptide at the C terminus of NS5A domain I to form a complex that is required for IRES-mediated viral protein translation (Khachatoorian et al., 2012b).

Considering the significant homology between HSC70 and HSP70, we proceeded to analyze the NS5A/HSC70 interaction biochemically. Recombinant full-length HSC70 and full-length NS5A were expressed in and purified from bacteria and were tested for binding through surface plasmon resonance (SPR) analyses. Both proteins were expressed in fusion with maltose binding protein (MBP). Full-length NS5A was immobilized on the SPR chip, and HSC70 was injected at different concentrations to assay its binding. As negative control for NS5A binding, human serum albumin (HSA) was injected at the same concentrations. The binding signal for HSA was subtracted from HSC70 binding in order to set HSA binding signal to zero response units, and the resultant HSC70 signal was plotted. As shown in Fig. 1A, HSC70 directly bound NS5A, and the dissociation constant was determined to be $\sim 2.22 \times 10^{-6}$.

NS5A is known to be a promiscuous protein that can bind to multiple host proteins. To further confirm that the observed interaction between HSC70 and NS5A is specific, we randomly selected human insulin as a negative control for binding to NS5A. Different concentrations of human insulin were tested for NS5A interaction. As shown in Fig. 1B, no significant interaction above background binding was observed between insulin and NS5A. We also tested the binding of insulin to HSP70 and did not see any interaction (Fig. 1C).

The NS5A/HSC70 interaction is mediated with both the nucleotide binding domain (NBD) and the substrate binding domain (SBD) of HSC70

We previously showed that the NS5A/HSP70 interaction is mediated by the NBD of HSP70 (Khachatoorian et al., 2012b). To further characterize the NS5A/HSC70 interaction, we also expressed and purified recombinant MBP-fusion HSC70-NBD (N-terminal) and HSC70-SBD (the entire C-terminal fragment of HSC70) and tested them in SPR assays for binding to full-length NS5A as above. We found that NS5A directly binds both HSC70-NBD and HSC70-SBD (Fig. 1D and E), and the dissociation constants were determined to be $\sim 2.9 \times 10^{-6}$ for HSC70-NBD and $\sim 7.54 \times 10^{-6}$ for HSC70-SBD.

HSC70 and HSP70 do not bind each other directly in vitro

Considering the fact that both HSC70 and HSP70 directly bind NS5A and that both of these HSPs were found as NS5A binding proteins in our mass spectrometric analyses, we proceeded to examine the possibility that HSC70 and HSP70 could directly bind each other and potentially be involved in the formation of NS5A complex(es) with HSPs. We expressed recombinant full-length HSP70 (not in fusion with MBP) and tested its interaction with full-length HSC70 and its NBD and SBD. As shown in Fig. 1F–H, no interaction was detected between any of the HSC70 and HSP70 protein constructs. We also tested the binding of all HSC70 constructs with MBP-fusion HSP70-NBD and obtained similar results indicating no binding (data not shown).

HSC70 binds NS5A in vivo

After demonstrating a direct interaction between recombinant HSC70 and NS5A *in vitro*, we proceeded to determine whether this interaction occurs *in vivo* in the HCV cell culture (HCVcc) system. Huh-7.5 cells were infected for 72 h. Subsequently, the infected cells and the control uninfected cells were harvested. The lysates were subjected to immunoprecipitation with antibody against

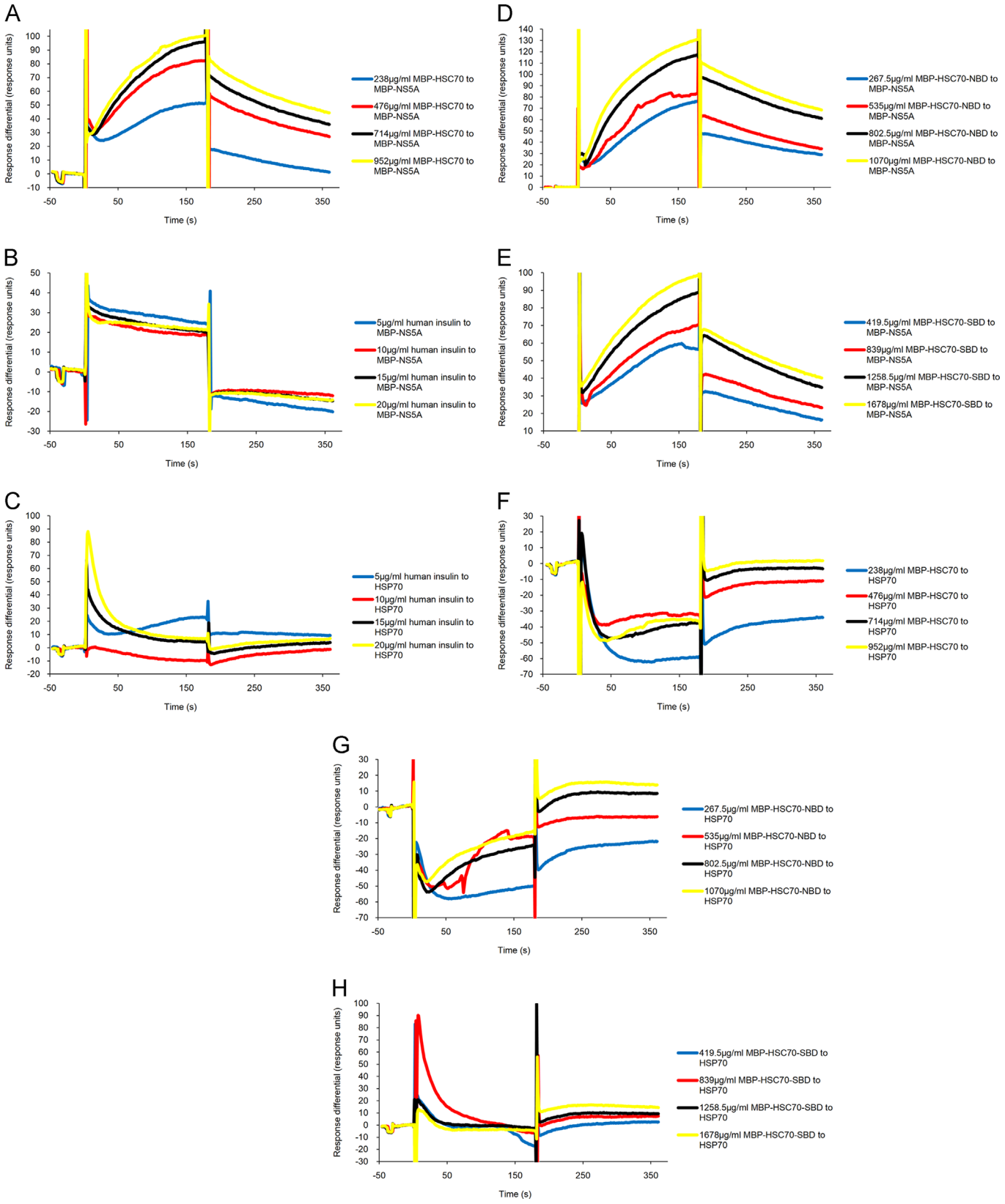


Fig. 1. HSC70 directly binds NS5A *in vitro* through both its nucleotide binding domain (NBD) and substrate binding domain (SBD) and does not bind HSP70. All panels display surface plasmon resonance (SPR) analyses. The immobilized and injected proteins are indicated in figure legends. For all assays, the injection time is 180 s followed by 180 s of dissociation. In panels (A) and (D–H), the binding signal for human serum albumin (HSA) (negative control) is subtracted from the binding of HSC70 constructs (*i.e.* HSA binding is set at zero response units). (A) Full-length HSC70 directly binds NS5A. (B) Human insulin does not bind to NS5A. (C) Human insulin does not bind HSP70. (D) HSC70-NBD directly binds NS5A. (E) HSC70-SBD directly binds NS5A. (F) Full-length HSC70 does not bind HSP70. (G) HSC70-NBD does not bind HSP70. (H) HSC70-SBD does not bind HSP70.

HSC70 and the corresponding IgG as negative control, and the presence of coimmunoprecipitated NS5A was assays by Western analyses with antibody against NS5A. As shown in Fig. 2, NS5A was coimmunoprecipitated from infected cells with antibody against HSC70.

HSC70 and HSP70 do not bind each other in vivo

The presence of HSP70 in the above coimmunoprecipitation assay was assayed by Western analyses with antibody against HSP70, and no coimmunoprecipitated HSP70 was detected in the infected cells (Fig. 2).

HSC70 and NS5A colocalize in vivo

After confirming the NS5A/HSC70 interaction *in vivo* biochemically, we further investigated this interaction *in vivo* by performing immunofluorescence analyses in infected cells. Cells were infected for 48 h and immunofluorescence was performed by antibodies against HSC70 and NS5A. As shown in Fig. 3, NS5A almost entirely colocalized with HSC70 (M1 = 0.9766), and HSC70 mostly colocalized with NS5A as well (M2 = 0.7161).

RNAi-mediated knockdown of HSC70

In this study, we utilized siRNA against HSC70 (HSPA8 gene) to examine the role of HSC70 during viral proliferation. HSC70 is a very important housekeeping gene, so we first sought to determine if we could significantly knockdown HSC70 in our cell culture system with no cytotoxicity. Huh-7.5 cells were treated with siRNAs against HSC70, HSP70 (specifically the HSPA1A gene examined in our previous reports (Gonzalez et al., 2009; Khachatoorian et al., 2012a, 2012b)), or both or their corresponding control siRNAs, and MTT assays were performed every 24 h up to 96 h. (The siRNAs against HSC70 and HSP70 were obtained from different companies and, therefore, required their own control siRNAs.) As shown in Fig. 4A, no cytotoxicity was observed up to 96 h after siRNA treatment. As control, treatment with luteolin (a potential anti-cancer drug) resulted in excessive cellular toxicity (Fig. 4A). Knockdown of both HSC70 and HSP70 also did not result in any detectable cytotoxicity (Fig. 4A).

Subsequently, we performed Western analyses to determine the levels of HSC70 protein after siRNA treatment. A significant reduction in HSC70 protein levels was observed with HSC70 siRNA treatment (Fig. 4B and C). Interestingly, we observed upregulation of HSP70 with HSC70 knockdown (Fig. 4B and C). As control, knockdown of HSP70 abolished HSP70 protein (Fig. 4B and C). Simultaneous knockdown of both HSC70 and HSP70 also resulted in significant reduction of HSC70 and eliminated HSP70 protein (Fig. 4B and C).

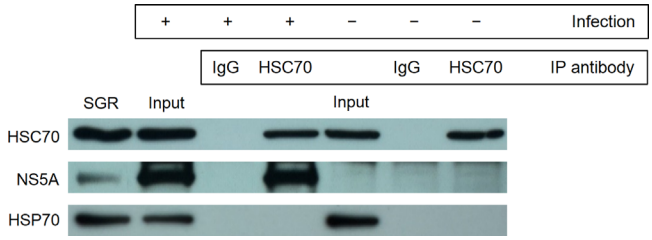


Fig. 2. HSC70 interacts with NS5A *in vivo*. Coimmunoprecipitation assay demonstrates that HSC70 binds NS5A in infected cells. Huh-7.5 cells were infected for 72 h and harvested. Cell lysates from infected and uninfected control cells were immunoprecipitated with antibody against HSC70 followed by Western analysis.

Knockdown of HSC70 significantly decreases HCV production

Next, we tested the anti-viral effects of HSC70 knockdown in the HCVcc system. Huh-7.5 cells were treated with siRNAs against HSC70, HSP70 or both as well as their corresponding control siRNAs. Cells were infected with the *Renilla* reporter virus and luciferase activity determined.

Knockdown of HSC70 resulted in a significant decrease in intracellular virus in agreement with previously reported results (Parent et al., 2009) (Fig. 5A). As expected, knockdown of HSP70 also attenuated intracellular virus as reported before (Gonzalez et al., 2009) (Fig. 5A). Importantly, simultaneous knockdown of HSC70 and HSP70 resulted in lower levels of intracellular virus compared with individual knockdowns (Fig. 5A). These results indicate potentially additive effects of knockdown of HSC70 and HSP70 and suggest that HSC70 and HSP70 have differential functions within the HCV life cycle.

Levels of viral secretion were also determined by infecting naïve cells with the supernatants of the above cell culture. As shown in Fig. 5B, individual knockdowns of HSC70 and HSP70 resulted in significant reduction of infectious virus production. However, the decrease in virus production observed with knockdown of HSC70 was much more than the effects of HSP70 knockdown. In addition, knockdown of both HSC70 and HSP70 resulted in a dramatic (over 95%) reduction in virus production again demonstrating additive effects (Fig. 5B).

To verify knockdown of HSC70 and HSP70 and their effects on virus production, we also utilized short hairpin RNA (shRNA) against HSC70 and HSP70 and obtained similar target knockdown (Fig. 6A and B) and subsequent effects on virus production (Fig. 6C).

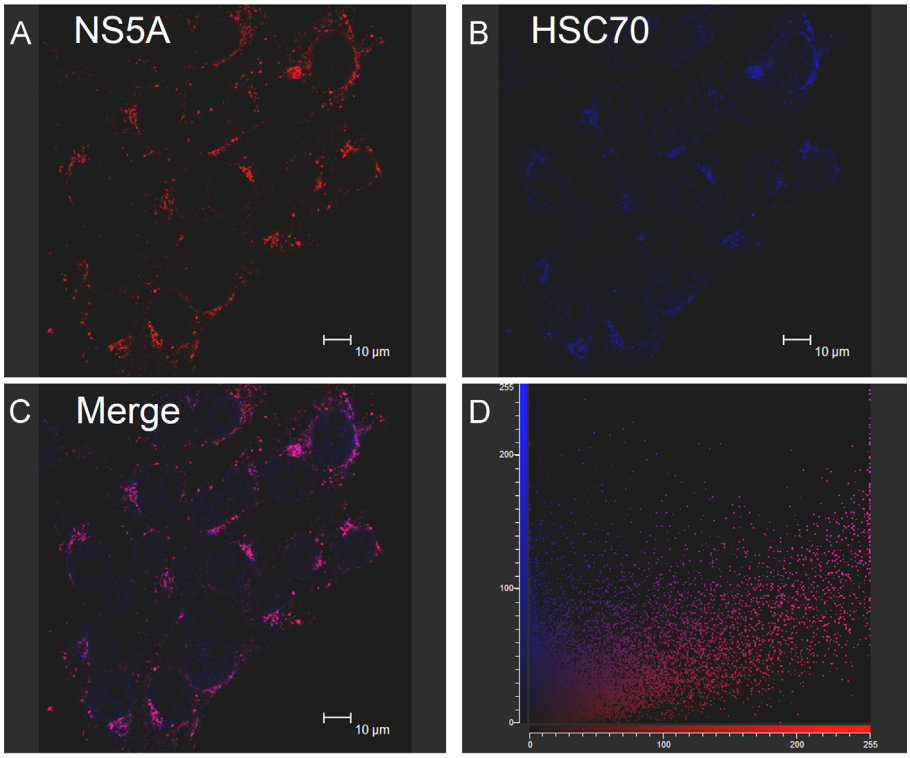
HSC70 is not involved in HCV RNA replication

After achieving a significant decrease in virus production by HSC70 knockdown, we proceeded to determine the specific phase (s) of viral life cycle affected by knockdown of HSC70 using the HCVcc system. Cells were infected for 24 h (to limit the duration of the assay to one viral life cycle), at which point the cells were harvested and the levels of positive strand viral RNA were determined by quantitative reverse transcriptase PCR (qRT-PCR).

As shown in Fig. 7A, knockdown of HSC70 did not reduce viral RNA levels compared with the corresponding control siRNA treatment. Instead, a slight, but statistically significant increase in viral RNA levels was observed. We believe that this increase may be due to increased viral proteins secondary to the compensatory increase in HSP70 (resulting in increased IRES mediated viral protein translations) seen with HSC70 knockdown (Figs. 4B and C, and 6A and B). However, as shown in Fig. 7A, knockdown of HSP70 resulted in a significant decrease in viral RNA levels potentially due to decreased viral protein translation as reported previously (Gonzalez et al., 2009) and in this study (see below). As a negative control, treatment of cells with Brefeldin A (a known inhibitor of secretion (Goldwasser et al., 2011)) did not affect viral RNA levels, while treatment with 2'-C-methyl-cytidine, a previously reported inhibitor of the viral NS5B polymerase (Le Pogam et al., 2006), significantly decreased viral RNA replication levels (Fig. 7A).

HSC70 is not involved in regulating NS5A-augmented IRES-mediated translation

We also determined the levels of NS5A-augmented IRES-mediated translation in a previously described virus-free cell culture-based bicistronic reporter system (Gonzalez et al., 2009). This reporter consists of a *Renilla* luciferase (RLuc) open reading frame (ORF) and a *Firefly* luciferase (FLuc) ORF driven by a 5'-cap



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	M1	M2
	0.9512	0.7827
	0.9776	0.6911
	0.9934	0.6811
	0.9842	0.7095
Mean	**0.9766**	**0.7161**
stdev	**0.018131**	**0.045932**

Fig. 3. HSC70 colocalizes with NS5A *in vivo*. Immunofluorescence assay demonstrates colocalization of HSC70 and NS5A *in vivo*. Huh-7.5 cells were infected for 48 h followed by simultaneous immunofluorescence staining with antibodies against HSC70 and NS5A. (A) HSC70 (blue). (B) NS5A (red). (C) Immunofluorescence staining for both HSC70 and NS5A (pink). (D) Scatter plot demonstrating the colocalization of HSC70 and NS5A. (E) Table indicating Manders overlap coefficients for colocalization of HSC70 with NS5A (M1) and colocalization of NS5A with HSC70 (M2). Overlap analyses were done on four independent immunofluorescence experiments.

and the HCV IRES, respectively (Fig. 7B). The ratio of *Firefly* to *Renilla* luciferase values (FLuc/RLuc) reflects the levels of HCV IRES-mediated translation. We have previously shown that knockdown of HSP70 as well as treatment with quercetin, an HSP synthesis inhibitor, decreases the NS5A-driven increase in IRES-mediated translation (Gonzalez et al., 2009; Khachatoorian et al., 2012a).

As expected, knockdown of HSP70 dramatically inhibited the NS5A-driven IRES activity (Fig. 7C). Treatment with HSC70 siRNA, however, did not inhibit, but resulted in a slight increase in NS5A-augmented IRES-mediated translation in agreement with the results of viral protein production assays below (Fig. 8).

HSC70 is not involved in viral protein production

To determine the effect of HSC70 on viral protein production, following siRNA treatment, cells were infected up to 28 h, and intracellular viral protein production was determined at several time points during the 28 h infection window. As shown in Fig. 8A, treatment with the control siRNAs resulted in a steady increase in viral protein levels during the first 24 h followed by a large increase after 24 h at time point 28 similar to our previously reported results (Khachatoorian et al., 2012a) suggesting that the duration of viral life cycle is approximately 24 h.

As expected, treatment with HSP70 siRNA decreased viral protein levels throughout the time course (Fig. 8). This is in agreement with our previously reported data implicating HSP70 in regulating viral translation (Gonzalez et al., 2009; Khachatoorian et al., 2012a, 2012b). A significant reduction in viral protein levels was also observed after the first 24 h with knockdown of HSP70 (Fig. 8).

Treatment with HSC70 siRNA, on the other hand, did not decrease intracellular viral protein levels; instead, a slight, but statistically significant increase was observed (Fig. 8). As mentioned above, this could be caused by a compensatory effect of increased HSP70 levels in response to HSC70 knockdown. Intracellular viral protein production, however, was reduced after the first 24 h indicating a decrease in the levels of secreted virions which results in infection of fewer cells after the first viral life cycle (Fig. 8A).

We considered the possibility that the compensatory upregulation of HSP70 caused by knockdown of HSC70 could mask a potential role for HSC70 in viral protein translation. To address this possibility, we also simultaneously knocked down both HSC70 and HSP70, thereby eliminating the compensatory upregulation of

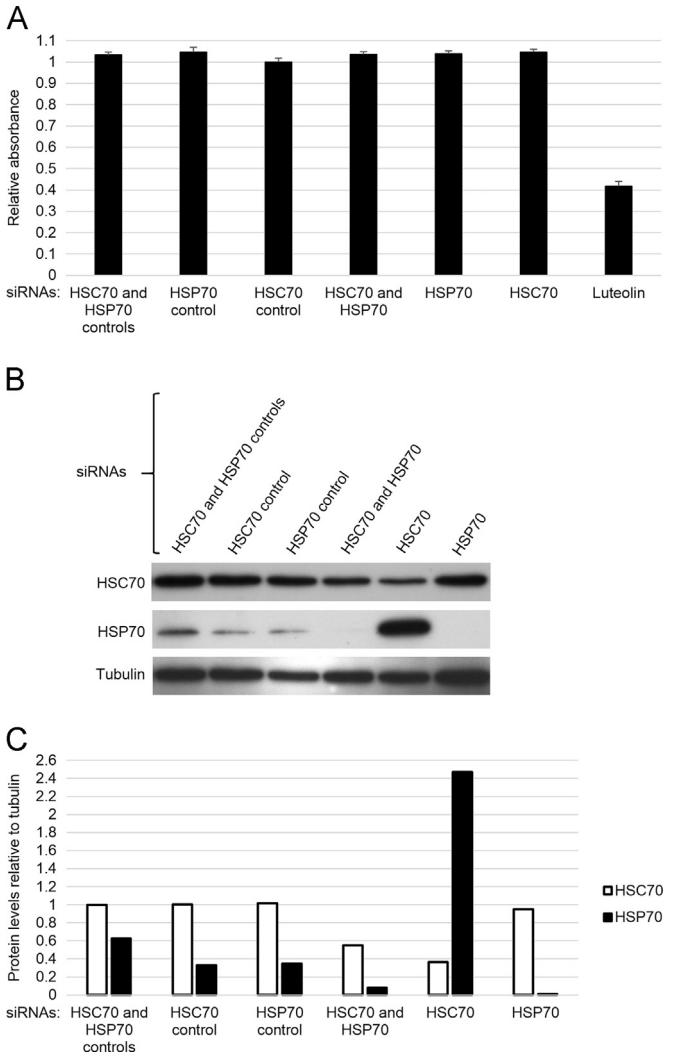

Fig. 4. siRNA-mediated knockdown of HSC70 is not cytotoxic to cells. (A) MTT assay demonstrates that knockdown of HSC70 does not have any cytotoxic effects at 96 h after siRNA transfection. (B) Western analysis of cells treated with the above siRNAs. (C) Densitometry of the Western blot in panel B.

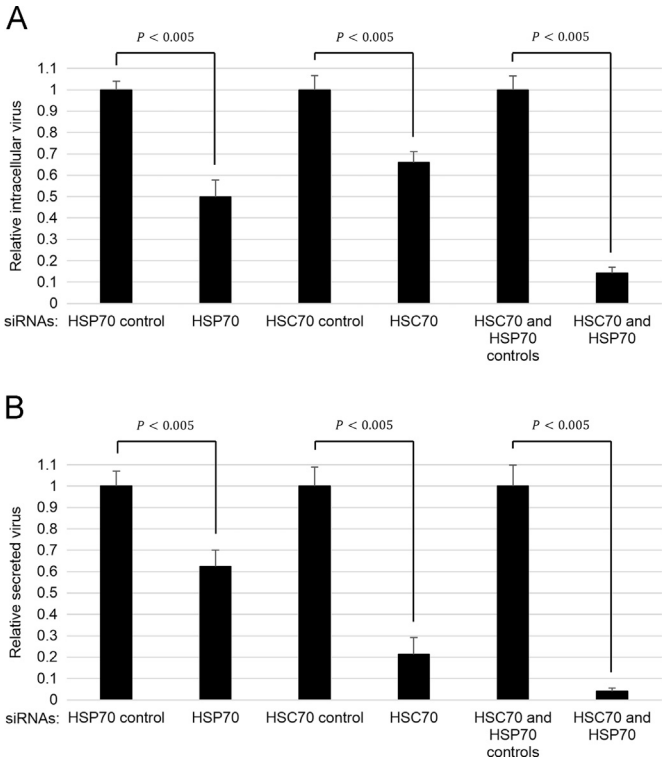

Fig. 5. siRNA-mediated knockdown of HSC70 significantly decreases virus production. (A) Intracellular virus production assay demonstrates that knockdown of HSC70 results in a significant decrease in intracellular virus levels. (B) Long term infectious virion secretion assay indicates that knockdown of HSC70 dramatically reduced infectious virion secretion.

HSP70. As shown in Fig. 8, knockdown of both HSC70 and HSP70 resulted in decreased viral protein levels during the first 24 h similar to knockdown of HSP70 alone. Furthermore, there is a highly significant decrease in viral protein levels after 24 h compared with each individual knockdown. These observations suggest that HSC70 and HSP70 have distinct roles in the viral life cycle and that HSC70 is unlikely to be involved in viral protein translation.

The fact that viral RNA levels are not decreased by HSC70 as well as the interesting observation that viral protein levels decrease after one viral life cycle despite no decrease during the life cycle strongly suggested to us that HSC70 is involved in regulating point(s) of viral life cycle after viral protein translation, *i.e.* virion assembly or viral secretion.

Knockdown of HSC70 significantly impairs intracellular infectious virion assembly

We next determined the effect of knockdown of HSC70 on viral assembly. Following siRNA treatment and infection, intracellular infectious virus was obtained by freeze/thaw treatment and used to infect naïve cells. As shown in Fig. 9A, levels of intracellular infectious virions were reduced by RNAi-mediated knockdown of HSC70 and HSP70. In contrast to the intracellular infectious virus results, a slight increase in viral protein levels was seen with knockdown of HSC70, whereas protein levels were reduced with HSP70 knockdown (Fig. 8B and data not shown). These results implicate HSC70 in viral assembly and the decreased infectious intracellular virus seen with HSP70 is likely to be related to lowered viral protein rather than a direct effect on assembly.

We also collected secreted virus in a 5-hour window of time, determined the levels of secreted virus, and obtained similar results to the above assembly assay (Fig. 9B). These results strongly suggest that the decrease in levels of secreted virus observed with knockdown of HSC70 is most likely caused by an assembly defect rather than a secretion defect.

Discussion

We have previously identified HSC70 to be part of a complex of proteins that include NS5A and host proteins HSP70 and HSP40 (Gonzalez et al., 2009). We have demonstrated the significance of the NS5A/HSP70 interaction for its role in HCV IRES-mediated translation (Gonzalez et al., 2009; Khachatoorian et al., 2012a, 2012b). Considering the significant homology between HSP70 and HSC70, we have been highly interested in further studying the NS5A/HSC70 interaction. It has also been reported that HSC70 is part of the HCV viral particles, and HSC70 was implicated in modulation of assembly or secretion (Parent et al., 2009). In this study, we further analyzed the NS5A/HSC70 interaction biochemically and conducted detailed analyses of the role of HSC70 in various phases of the viral life cycle.

Our lab and others have previously shown the NS5A/HSC70 interaction (Chen et al., 2010; Gonzalez et al., 2009). Due to the use of cellular systems, however, it was not determined whether NS5A directly binds HSC70. In this study, we have shown for the first

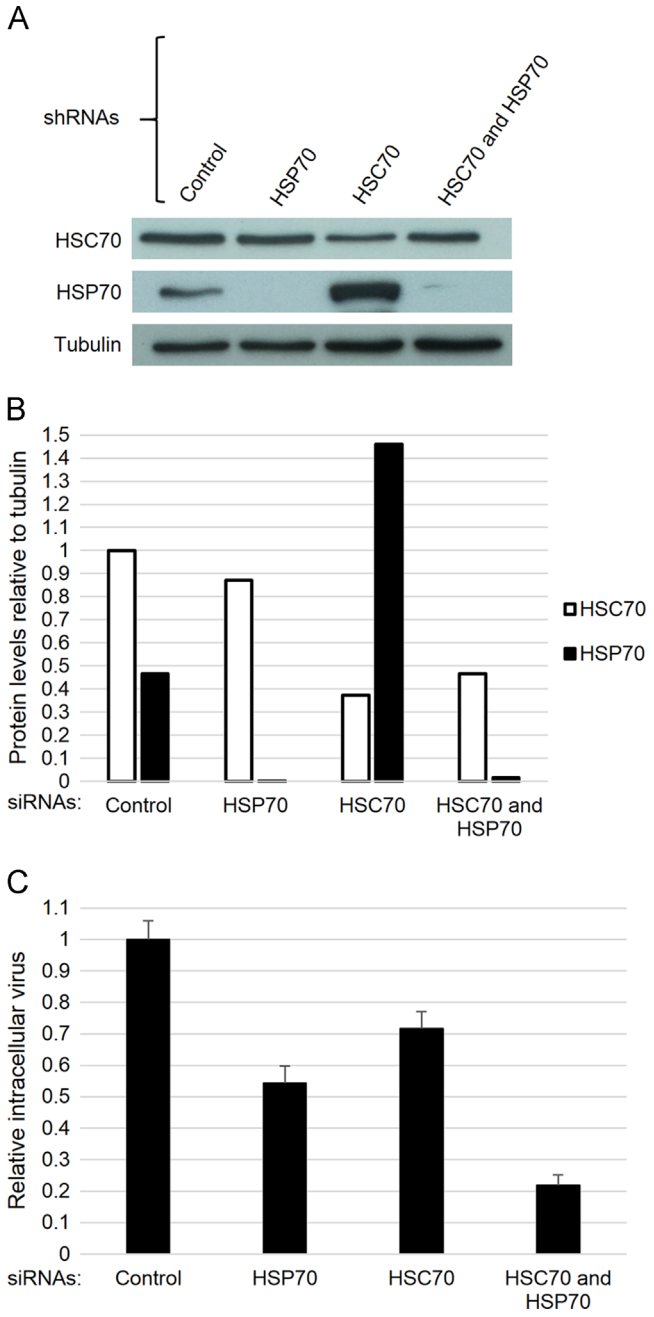

Fig. 6. shRNA-mediated knockdown of HSC70 significantly decreases virus production. (A) Western analysis of cells treated with shRNAs against HSC70, HSP70, or both. (B) Densitometry of the Western blot in panel (A). (C) Intracellular virus production assay demonstrates that knockdown of HSC70 results in a significant decrease in intracellular viral levels.

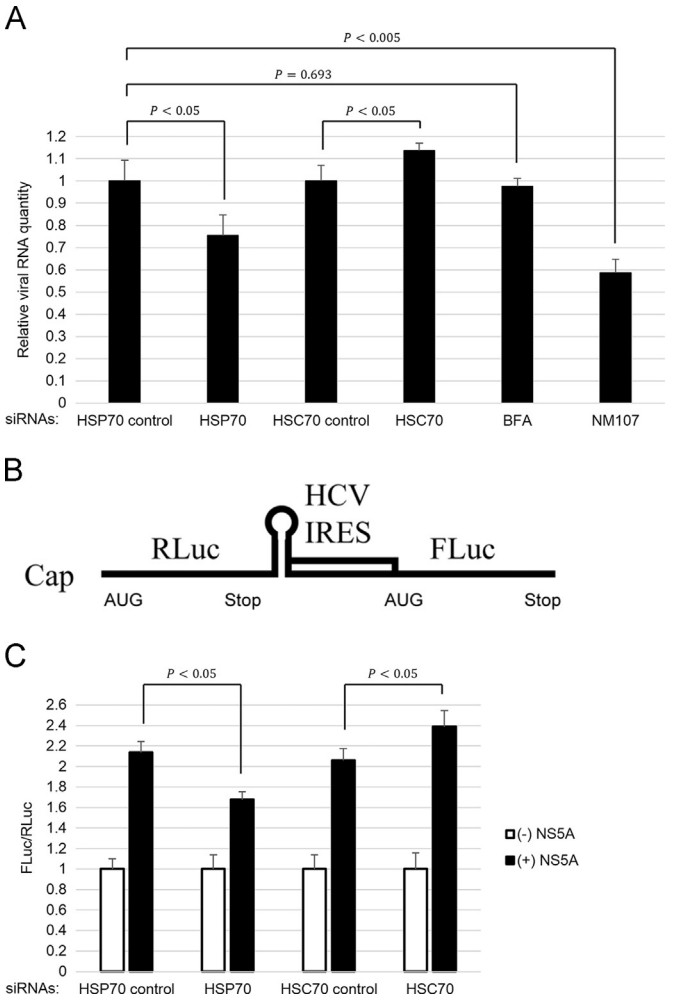

Fig. 7. Knockdown of HSC70 does not affect viral RNA replication and NS5A-augmented IRES-mediated translation. (A) Quantitative reverse transcriptase PCR (qRT-PCR) analysis indicates that knockdown of HSC70 does not affect viral RNA replication. (B) Schematic of the bicistronic reporter construct used in the dual luciferase assays in part (C). This construct contains the *Renilla* luciferase and *Firefly* luciferase ORFs under the control of a 5'-cap and the HCV IRES, respectively. The ratio of *Firefly* to *Renilla* measurements is indicative of IRES-mediated translation. (C) IRES assay indicates that knockdown of HSC70 does not decrease NS5A-augmented IRES-mediated translation.

time that HSC70 directly binds NS5A *in vitro* through surface plasmon resonance (SPR) analyses utilizing purified recombinant proteins, suggesting that no other viral or host protein is required for the NS5A/HSC70 interaction. We have further confirmed the NS5A/HSC70 interaction *in vivo* by performing coimmunoprecipitation assays on infected cells where we were able to successfully pull down NS5A with antibody against HSC70. We also demonstrated that NS5A and HSC70 colocalize in infected cells *in vivo* by immunofluorescence analyses.

We further showed, through SPR analyses, that both the nucleotide binding domain (NBD) and the substrate binding domain (SBD) of HSC70 directly bind NS5A in contrast with the HSP70 interaction with NS5A which we have previously shown to involve HSP70-NBD alone (Khachatoorian et al., 2012b). We speculate that this difference is caused by the fact that while HSC70 and HSP70 are 86% identical to each other, the C terminus of the HSP70 family of proteins bear the least homology to each other (Daugaard et al., 2007). We are currently investigating the direct binding of the three NS5A domains to HSC70 to better understand the mechanism of this interaction.

To find out whether HSC70 and HSP70 could directly bind each other and be potentially involved in the formation of NS5A complex(es) with heat shock proteins, we utilized SPR analyses and did not find any interaction between HSC70 and HSP70 *in vitro*. In addition, we did not detect any interaction between HSC70 and HSP70 in infected cells *in vivo* as no HSP70 was detected in our coimmunoprecipitation analyses with antibody against HSC70. These findings are in agreement with our identification of the distinct roles of HSC70 and HSP70 within the viral life cycle in this report and previously (Gonzalez et al., 2009) and further suggest that the NS5A/HSC70 and the NS5A/HSP70 complexes form independently of each other to facilitate different functions within different stages of the viral life cycle.

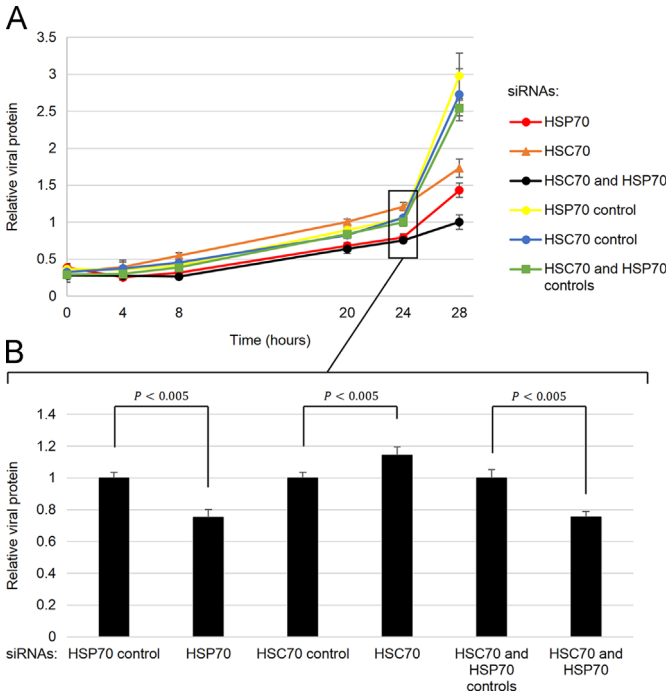

Fig. 8. Knockdown of HSC70 does not affect intracellular viral protein production. (A) Intracellular viral protein production assay indicates that knockdown of HSC70 does not decrease intracellular viral protein production. Huh-7.5 cells were treated with siRNAs against HSC70, HSP70, or their corresponding control siRNAs and infected with the reporter virus 24 h later. At the indicated time points after infection, cells were harvested and luciferase activity was measured. (B) Bar graph corresponding to the 24 h time point of the above assay.

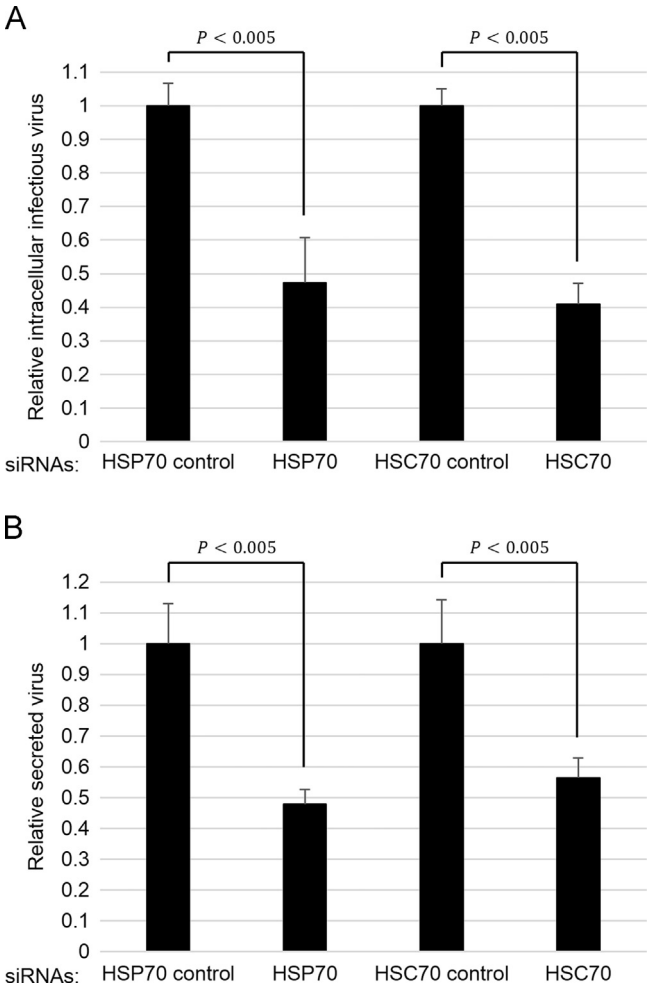

Fig. 9. Knockdown of HSC70 significantly reduces intracellular infectious virion assembly. (A) Intracellular infectious virion assembly assay demonstrates that knockdown of HSC70 significantly inhibits virion assembly. (B) Short term infectious virion secretion assay demonstrates the effect of knockdown of HSC70 on virus secretion.

We determined the anti-viral activity of HSC70 knockdown in our HCV cell culture (HCVcc) system utilizing the *Renilla* reporter virus. Knockdown of HSC70 resulted in a significant decrease in virus production which was comparable to the reduction of virus production observed by knockdown of HSP70 in this study and before (Gonzalez et al., 2009). Considering the significant homology between HSC70 and HSP70 and their potential compensatory regulation as seen in Fig. 4B and 6A, we asked whether knockdown of HSC70 or HSP70 displays anti-viral effects by affecting the same or different phase(s) of viral life cycle. We reasoned that if HSC70 and HSP70 are involved in regulating different phase(s) of viral life cycle, we would expect to see an additive anti-viral effect if both HSC70 and HSP70 are knocked down, whereas no significant additive effect should be expected otherwise. Simultaneous knockdown of both HSC70 and HSP70 indeed displayed a dramatic and additive inhibitory effect suggesting that they potentially play distinct roles in the viral life cycle.

To obtain further insights into the role of HSC70 and HSP70 in the viral life cycle, we measured secreted virus levels. These results revealed that knockdown of HSC70 impacts secretion significantly more than knockdown of HSP70 in contrast with the intracellular virus levels. This occurs despite significant upregulation of HSP70 as a result of knockdown of HSC70 indicating distinct functions for HSC70 and HSP70 during the viral life cycle.

Intracellular infectious virion assembly was significantly inhibited by knockdown of HSC70. Knockdown of HSP70 also resulted in a significant decrease in intracellular infectious virus levels most likely due to decreased viral protein production levels. We considered the possibility that HSC70 may play a role in earlier stages of viral life cycle, (*i.e.* viral genome replication and viral protein translation), which may be masked by the upregulation of HSP70 due to knockdown of HSC70. Viral protein translation levels significantly increased with knockdown of HSC70, which we attribute to the compensatory increase in HSP70 levels seen by HSC70 knockdown. However, we observed that simultaneous knockdown of HSC70 and HSP70 does not display the compensatory upregulation of HSP70 and results in decreased viral translation similar to knockdown of HSP70 alone suggesting that HSC70 likely does not play a role in viral protein translation. The results of double knockdown of HSC70 and HSP70 also suggest that viral genome replication is also unlikely to be mediated by HSC70. If HSC70 was involved in viral genome replication, we would expect to observe a higher impact on viral protein translation levels with double knockdown of HSC70 and HSP70. Thus, our results are consistent with the notion that while HSP70 plays a role in viral translation, HSC70 most likely is involved in the assembly of infectious virus particles.

These findings as well as the fact that HSC70 and HSP70 are host proteins open up potential therapeutic avenues to treat HCV infection by targeting host proteins as opposed to viral proteins to minimize the development of resistance.

Materials and methods

Plasmid constructs

All recombinant proteins were cloned in the pET-28b plasmid (Novagen, 69865-3). The HCV IRES reporter plasmid and the NS5A

and GFP retroviral expression vectors pMSCV-NS5A-FLAG and pMSCV-GFP, respectively, have been previously described (Gonzalez et al., 2009). An intragenotype 2 chimeric monocistronic reporter virus, pNRLFC based on pJ6/JFH-C parental virus has been described previously (Arumugaswami et al., 2008). For the current study, we have used a chemically synthesized plasmid pFNX-RLuc (having similar sequences to pNRLFC) for construction of recombinant virus.

Production and purification of recombinant proteins

All proteins were expressed and purified as described previously (Wheatley et al., 2013). Briefly, protein expression was induced with 1 mM IPTG in BL21(DE3) *Escherichia coli* cells, shaking at 250 rpm, for 5 h at 37 °C. Cells were centrifuged for 5 min at 6000 rpm and stored at −20 °C. Cells were resuspended in 50 mM Tris–HCl pH 7.6, 300 mM NaCl, and 20 mM imidazole, with Protease Inhibitor Cocktail (Sigma-Aldrich, P8849-5ml) and lysed by sonication. Cells were spun down in rotor SS-34 at 16,500 rpm for 30 min, filtered through a 0.2 μm filter, and applied to a Hi-trap Nickel column by a syringe at room temperature. Proteins were eluted in one step with 50 mM Tris–HCl pH 7.6, 300 mM NaCl, and 400 mM imidazole.

Surface plasmon resonance (SPR)

Binding studies were performed on a Biacore 3000 instrument (Biacore AB, Uppsala, Sweden). Proteins were immobilized on CM5 sensor chips by amine coupling. The solution phase analytes were dissolved in HBS-EP buffer, which contained 0.15 M NaCl, 10 mM HEPES, pH 7.4, 3 mM EDTA, and 0.005% polysorbate 20. The solutions traversed the sensors at a flow rate of 50 μl/min. Costar low-retention polypropylene tubes (Corning, 3207) were used throughout. Binding results were expressed in resonance units. Kinetics and dissociation constants were analyzed and calculated with BIAevaluation Software Version 4.1.

Cell culture

Cell lines huh-7.5, SGR (an HCV subgenomic replicon harboring cell line), and 293T were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C in Dulbecco's Modified Eagle Medium (Mediatech, 10-013-CM) supplemented with 10% fetal bovine serum (Omega Scientific, FB-01) and 2 mM L-glutamine (Life Technologies, 25030). Huh-7.5 cells were a kind gift from Charles Rice (The Rockefeller University, New York, NY). SGR cells were previously described (Blight et al., 2002). 293T cells were purchased from ATCC (CRL-11268).

Antibodies

HSC70 (Santa Cruz Biotech, sc-7298), HSP70 (Santa Cruz Biotech, sc-66048), mouse IgG (Santa Cruz Biotech, sc-2025), Alexa Fluor 647-conjugated donkey anti-mouse IgG (H+L) (Life Technologies, A31571), Alexa Fluor 594-conjugated donkey anti-rabbit IgG (H+L) (Jackson ImmunoResearch, 711-585-152), and tubulin (abcam, ab6160).

Coimmunoprecipitation

Huh-7.5 cells were infected for 72 h and harvested. Subsequently, the lysates from infected and control uninfected cells were utilized for coimmunoprecipitation assays with antibody against HSC70 and mouse IgG as negative control. Coimmunoprecipitations were done using Protein A/G PLUS-Agarose (Santa Cruz Biotech, sc-2003) according to manufacturer's instructions.

Immunofluorescence

Huh-7.5 cells were grown on glass cover slips and infected for 48 h. Subsequently, cells were briefly rinsed with PBS, fixed for 15 min at room temperature with 4% paraformaldehyde in PBS, pH 7.4, permeabilized for 3 min at room temperature with 0.1% Triton X-100 and 1% 2.5 M sucrose in PBS, and blocked for 1 h at room temperature with 10% serum in PBS, pH 7.4. Then cells were washed with PBS and incubated with antibodies against HSC70 (1:100) and NS5A (1:100) in 1% BSA in PBS for 2 h. After washing, the cells were double stained with Alexa Fluor 647- and Alexa Fluor 594-conjugated secondary antibodies.

Confocal microscopy

The cover slips prepared above were mounted on microscope slides with VECTASHIELD Mounting Medium (Vector Laboratories, H-1000), and immunofluorescence was detected by a Leica TCS SP2-FILM confocal microscope. Colocalization was analyzed with ImageJ v1.47v software according to software instructions.

RNA interference

siRNAs against HSC70 (Santa Cruz Biotech, sc-29349) and HSP70 (Thermo Scientific, M-005168-01-0005) as well as Control siRNA-A (Santa Cruz Biotech, sc-37007) and siGENOME Non-Targeting siRNA Pool #2 (Thermo Scientific, D-001206-14-05) were transfected into huh-7.5 cells using Lipofectamine 2000 Transfection Reagent (Life Technologies, 11668-019) according to manufacturer's instructions. shRNAs against HSC70 (Santa Cruz Biotech, sc-29349-SH) and HSP70 (Santa Cruz Biotech, sc-29352-SH) as well as control shRNA plasmid-A (Santa Cruz Biotech, sc-108060) were transfected into huh-7.5 cells using X-tremeGene 9 DNA Transfection Reagent (Roche, 6365779001) according to manufacturer's instructions.

Cell viability

Cell viability was determined using MTT Cell Proliferation assay (ATCC, 30-1010K) according to manufacturer's instructions.

Densitometry

Western blot images were analyzed by ImageJ v1.47v software according to software instructions.

Infectious virus production

pFNX-RLuc was *in vitro* transcribed, and the purified RNA was electroporated into huh-7.5 cells to generate infectious viral supernatant as previously described (Arumugaswami et al., 2008).

Viral assays

The HCV reporter virus was utilized as described previously (Khachatoorian et al., 2012a). All infection assays were performed with a viral titer of 10^3 focus forming units and a multiplicity of infection (MOI) of 0.1. Intracellular virus production: huh-7.5 cells were infected for 3 h at which point the medium was replaced and infection was allowed to proceed. Cells were harvested 48 h later, and luciferase activity was measured using the *Renilla* Luciferase Assay System (Promega, E2820). Intracellular viral protein production: huh-7.5 cells were infected for 3 h at which point the medium was replaced and infection was allowed to proceed. Cells were harvested at the indicated time points, and luciferase activity was measured. Infectious virion secretion: (1) Long term assay: the supernatants from the above cultures were used to infect naïve

cells for 3 h at which point the medium was replaced and infection was allowed to proceed. Cells were harvested 48 h after infection, and *Renilla* luciferase activity was measured. (2) Short term assay: supernatants were removed from original cultures, cells were washed with PBS, and fresh medium was added. Five hours later, the supernatants were collected and used to infect naïve cells for 3 h at which point the medium was replaced. Cells were harvested 72 h after infection, and luciferase activity was measured. Intracellular infectious virion assembly: huh-7.5 cells were infected for 3 h at which point the medium was replaced and infection was allowed to proceed. Supernatants were removed 24 h after infection, cells were washed with PBS, and fresh medium was added. The cultures were subjected to three cycles of freeze–thaw to release assembled viral particles. These suspensions were cleared of cellular debris and used to infect naïve cells for 3 h at which point the medium was replaced and infection was allowed to proceed. Cells were harvested 72 h after infection, and luciferase activity was assayed.

Quantitative reverse-transcriptase PCR

Huh-7.5 cells, plated in 10 cm plates, were treated with siRNAs against HSC70, HSP70 or the corresponding control siRNAs. Twenty-four hours after transfection, cells were infected with the *Renilla* reporter virus. Twenty-four hours after infection, cells were harvested, and total RNA was extracted using RNeasy Mini Kit (Qiagen, 74104). cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad, 1708891). Quantitative PCR was performed using the Applied Biosystems 7500 Fast Real-Time PCR System with 2x SYBR Green Master Mix (Diagenode, GMO-SG2x-A300) in 25 μL reactions. The real-time PCR cycling conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s each as well as a final dissociation stage of 95 °C for 15 s and 60 °C for 1 min. The primers for the viral genome were derived from the 5'-non-coding region and were CTGGGTCCTTTCTTGGATAA and CCTATCAGGCAGTACCACA. HCV RNA levels were normalized to the housekeeping gene actin using the primers CCAACCGCGAGAAGATGA and CCAGAGGCGTACAGGGATAG.

IRES reporter assay

293T cells were treated with siRNAs against HSC70, HSP70, or the corresponding control siRNAs. Twenty-four hours after siRNA transfection, cells were transfected with the HCV IRES reporter plasmid and either pMSCV-NS5A-FLAG or pMSCV-GFP. All transfections were done using X-tremeGene 9 Transfection Reagent (Roche, 06365779001). Seventy-two hours post-transfection, *Renilla* and *Firefly* luciferase activity were determined using Dual Luciferase Assay System (Promega, E1910).

Statistical analysis

Error bars reflect the standard deviation. *P* values were determined by the Student *t* test. All viral assays were done with six biological replicates for each individual assay.

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